1: Biochem Pharmacol 2000 Aug 15;60(4):471-8

Antioxidative properties of natural coelenterazine and synthetic methyl coelenterazine in rat hepatocytes subjected to tert-butyl hydroperoxide-induced oxidative stress.

Dubuisson ML, de Wergifosse B, Trouet A, Baguet F, Marchand-Brynaert J, Rees JF.

Unite;;-2 de Biologie Animale, Universite Catholique de Louvain, Croix de Sud, 4-5, B-1348, Louvain-la-Neuve, Belgium. dubuisson@bani.ucl.ac.be

Coelenterazine (CLZn; 3, 7-dihydro-2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-8-benzylimidazolo++ +[1 ,2-a]pyrazin-3-one), the substrate for bioluminescence reactions in many marine animals, is endowed with high antioxidant properties. This work investigated the antioxidative properties of CLZn in primary cultures of rat hepatocytes subjected to the oxidant tert-butyl hydroperoxide (t-BHP). Micromolar concentrations of CLZn increased survival and decreased lipid peroxidation in rat hepatocytes subjected for 6 hr to 2.5 x 10(-4) M t-BHP. However, the extent of protection was limited by a strong toxicity of CLZn (IC(50) = 6.9 x 10(-5) M). The presence of t-BHP increased the cellular toxicity of CLZn. Methyl coelenterazine (CLZm, 3, 7-dihydro-2-methyl-6-(p-hydroxyphenyl)-8 benzylimidazolo[1, 2-a]pyrazin-3-one), a synthetic analogue of CLZn, demonstrated excellent antioxidant properties, even at very low (3 x 10(-6) M) concentrations and was not toxic throughout most of its effective concentration range. CLZm proved far more effective than reference antioxidants such as Trolox C(R), alpha-tocopherol, BHT, and probucol. The assay of thiobarbituric reactive substances (TBARS) associated with cells and in the culture medium indicated that 10(-5) M CLZm provided a total protection against t-BHP-induced lipid peroxidation. This coelenterazine analogue could be used as a model compound for investigating the action mechanism of imidazolopyrazinones in mammalian hepatocytes.

Biochemical Pharmacology, Vol. 60, pp. 471–478, 2000.

ELSEVIER

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00359-2

Antioxidative Properties of Natural Coelenterazine and Synthetic Methyl Coelenterazine in Rat Hepatocytes Subjected to *tert*-Butyl Hydroperoxide-Induced Oxidative Stress

Marlène L. N. Dubuisson,† Bertrand de Wergifosse,* André Trouet,* Fernand Baguet,* Jacqueline Marchand-Brynaert‡ and Jean-François Rees**

*Unité de Biologie Animale and ‡Laboratoire de Chimie Organique de Synthèse, Université Catholique de Louvain, Croix de Sud, 4-5, B-1348 Louvain-la-Neuve, Belgium

ABSTRACT. Coelenterazine (CLZn; 3,7-dihydro-2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-8-benzylimidazolo[1,2-*a*]pyrazin-3-one), the substrate for bioluminescence reactions in many marine animals, is endowed with high antioxidant properties. This work investigated the antioxidative properties of CLZn in primary cultures of rat hepatocytes subjected to the oxidant *tert*-butyl hydroperoxide (*t*-BHP). Micromolar concentrations of CLZn increased survival and decreased lipid peroxidation in rat hepatocytes subjected for 6 hr to 2.5×10^{-4} M *t*-BHP. However, the extent of protection was limited by a strong toxicity of CLZn ($IC_{50} = 6.9 \times 10^{-5}$ M). The presence of *t*-BHP increased the cellular toxicity of CLZn. Methyl coelenterazine (CLZm, 3,7-dihydro-2-methyl-6-(*p*-hydroxyphenyl)-8 benzylimidazolo[1,2-*a*]pyrazin-3-one), a synthetic analogue of CLZn, demonstrated excellent antioxidant properties, even at very low (3×10^{-6} M) concentrations and was not toxic throughout most of its effective concentration range. CLZm proved far more effective than reference antioxidants such as Trolox C®, α-tocopherol, BHT, and probucol. The assay of thiobarbituric reactive substances (TBARS) associated with cells and in the culture medium indicated that 10^{-5} M CLZm provided a total protection against *t*-BHP-induced lipid peroxidation. This coelenterazine analogue could be used as a model compound for investigating the action mechanism of imidazolopyrazinones in mammalian hepatocytes. BIOCHEM PHARMACOL 60;4:471–478, 2000.

KEY WORDS. coelenterazine; imidazolopyrazinone; antioxidant; *tert*-butyl hydroperoxide; lipid peroxidation; hepatocytes

CLZn§ is an imidazolopyrazinone (Fig. 1a) acting as a luminescent substrate in many bioluminescent marine organisms [1]. Observing the widespread occurrence of CLZn, in both bioluminescent and non-bioluminescent animals [2], and the high reactivity of IMPZs with ROS [3], we proposed that CLZn could help cells to cope with oxidative stress [4]. The antioxidant properties of CLZn, which could have preceded its utilisation in light-emitting mechanisms during evolution, have been demonstrated in human fibroblasts subjected to the lipid peroxidation initiator *t*-BHP [4]. Acellular lipid peroxidation assays confirmed that CLZn possesses chain-breaking properties similar to that of α-tocopherol [4].

Due to their high metabolic functions, high content in cytochromes P450 (CYP 450), and their central role in the biotransformation of xenobiotics, hepatocytes are very much exposed to oxidative stress *in vivo*, as indicated by the great number of liver pathologies which have been linked to oxidative stress [5]. Oxidants may generate lipid hydroperoxides decomposing into alkoxyl and peroxyl radicals, which then can oxidise all cellular constituents [6].

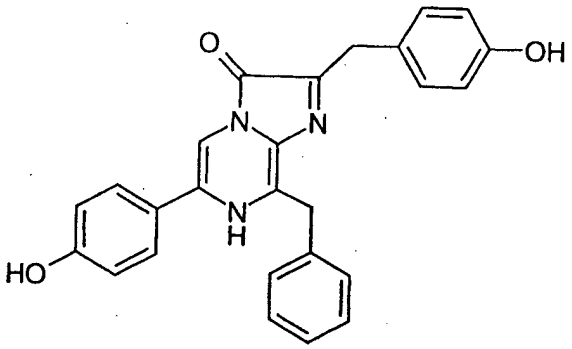
In this work, we investigated the antioxidant properties of CLZn and of a synthetic analogue (CLZm, Fig. 1b) possessing a methyl at the C-2 position on the fused imidazolopyrazinone ring in rat hepatocytes subjected to *t*-BHP. While being able to protect rat hepatocytes against the peroxide, CLZn proved to be rather toxic to these cells. On the other hand, micromolar concentrations of the analogue CLZm protected cells very effectively, with few signs of toxicity at higher doses. The results indicate that CLZm is a powerful antioxidant in cells and a good model compound for studies of the mechanisms underlying the antioxidant properties of IMPZs in rat hepatocytes.

† Corresponding author: Dr. Marlène Dubuisson, Unité de Biologie Animale and Laboratoire de Biologie Cellulaire, Croix du Sud 4-5, B-1348 Louvain-la-Neuve, Belgium. Tel. 32-10-473474; FAX 32-10-473515; E-mail: dubuisson@bani.ucl.ac.be

§ *Abbreviations:* BHT, butylated hydroxytoluene; CLZn, coelenterzaine; CLZm, methyl coelenterazine; CYP 450, cytochrome P450; DMEM, Dulbelcco's modified Eagle's medium F12 with HEPES and glutamax; IMPZ, imidazolopyrazinone; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; *t*-BHP, *tert*-butyl hydroperoxide; and WE, Williams' medium E.

Received 7 September 1999; accepted 30 December 1999.

a.



b.

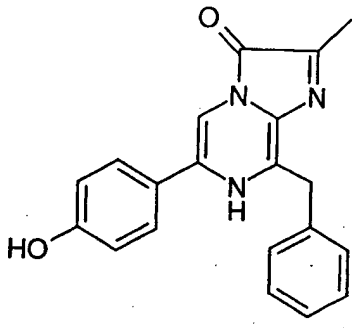


FIG. 1. Chemical structure of CLZn (a) and CLZm (b).

MATERIALS AND METHODS

Chemicals

BHT, TBA, MTT, 6-hydroxy-2,5,7,8-pentamethylchroman-2-carboxylic acid (Trolox C®), α-tocopherol, probucol, *t*-BHP, and collagenase (type IV, EC 3.4.24.3) were purchased from Sigma Chemical Co. The LDH assay kit was from Boehringer. Butanol was from Fluka while DMSO was obtained from Acros Organics. Coelenterazine (3,7-dihydro-2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one) and CLZm (3,7-dihydro-2-methyl-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one) were synthesised according to published methods [7]. Their purity was assessed through HPLC, ^{13}C-NMR, and ^{1}H-NMR. WE, DMEM F12 with HEPES and glutamax, and RPMI-1640 medium lacking phenol red were purchased from GIBCO BRL.

Cell Culture

Male Wister–Yops rats (averaging 200 g body weight) were obtained from Iffa Credo. Their hepatocytes, isolated according to the modified *in situ* collagenase liver perfusion technique of Seglen [8], were transferred to collagen-precoated culture plates (Cellon bovine dermal collagen, 60 μg/mL). The cells were seeded either on 96-well microplates (20,000 cells/well) for MTT and LDH-based experiments or on 6-well plates (1,000,000 cells/well) for the measurements of TBARS. Hepatocytes were first cultured for 4 hr at 37° (95% humidity, 5% CO_2) in WE medium containing 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin, and then the medium was changed to WE medium supplemented with antibiotics and additional factors (10 nM insulin, 0.1 μM dexamethasone, 10 μg/mL of a linoleic acid–BSA complex, 0.1 mM ascorbic acid, and 50 ng/mL of epidermal growth factor).

Oxidative Stress with t-BHP

Hepatocytes were incubated (37°, 95% humidity, 5% CO_2) for 6 hr with 2.5×10^{-4} M *t*-BHP in the presence or absence of the tested antioxidants. Antioxidants were solubilised in ethanol (final concentration < 1%) or dimethylformamide (DMF, final concentration < 0.03%) before addition to serum-free culture medium. Control experiments indicated that neither solvent influenced the cellular survival nor the peroxidation level of cellular lipids.

Measurements of Cellular Damage

SURVIVAL MEASURED BY THE MTT ASSAY. Cells were incubated in serum-free DMEM supplemented with 0.2 mg/mL of insulin and 0.25 mg/mL of BSA for 6 hr containing *t*-BHP and the tested antioxidants. Microplates were then rinsed with PBS. MTT (1 mg/mL in RPMI medium without phenol red) was added to the wells (50 μL/well). After a 2-hr incubation (37°, 95% humidity, 5% CO_2), plates were centrifuged (1400 *g*, 4 min, 4°), the medium was removed, and the formazan crystals formed within metabolically active cells were dissolved in DMSO (50 μL/well). Absorbance of formazan was read at 540 nm on a spectrophotometer (Titertek Multiskan, Labsystems). Blanks were read at 690 nm.

LDH RELEASE. Cells in 96-well plates were incubated for 6 hr with both *t*-BHP and the antioxidants in serum-free RPMI medium without phenol red. The release of LDH into the supernatant was then quantified by a colourimetric test based on NADH synthesis (cytotoxicity detection kit, Boehringer). Controls included untreated cells and cells lysed with Triton X-100 2% (100% mortality). The mortality of cells was calculated according to this scale.

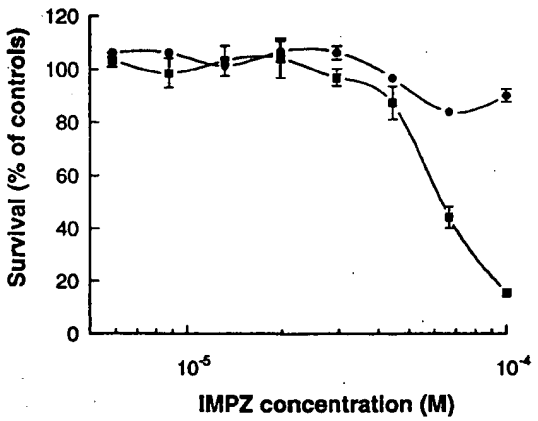


FIG. 2. Cytotoxicity curves of CLZn (■) or CLZm (●) applied on rat hepatocytes. Both IMPZs were applied for 6 hr and survival was measured by the MTT test. Each value is the mean ± SE of 6 replicates. Controls (100% survival) are cells treated with culture medium (DMEM) only.

Lipid Peroxidation Assay

The extent of lipid peroxidation was determined by quantifying the amount of TBARS in the medium and in cells. Treatments were carried out in 6-well plates containing serum-free RPMI medium without phenol red, as this could interfere with the assay. TBARS released into the culture medium were assayed according to the method of Wey *et al.* [9]. Briefly, a 500 µL aliquot of the supernatant was added to a mixture of 400 µL 15% TCA and 800 µL 0.67% (w/v) TBA and heated (95°) for 20 min in a water bath. After cooling, 3 mL of butanol was mixed thoroughly into the solution, tubes were centrifuged, and the fluorescence (excitation: 521 nm; emission: 552 nm) in the butanol phase was measured. TBARS in cells were determined according to Günther *et al.* [10]. TCA (2 mL, 2.5% w/v) was added to each rinsed plate and the cells were scraped off. After centrifugation (13,000 *g*, 2 min), 500 µL of the supernatant was taken for the reaction with the above TCA–TBA mixture and subsequent measurement of TBARS as described above. Standard curves specific for the assays in both compartments were created using malondialdehyde (MDA) and the signal converted into MDA equivalents per well. Control tests indicated that none of the tested antioxidants interfered with the assay.

Statistical Analysis

Significance of differences between treatments was tested by a one-way analysis of variance (ANOVA).

RESULTS

Cytotoxicity of CLZn and CLZm

First, toxicity curves for CLZn and CLZm on rat hepatocytes were established using the same conditions as those employed in the following experiments. The viability of hepatocytes treated for 6 hr with increasing concentrations (3×10^{-6}–10^{-4} M) of CLZn or CLZm was analysed (MTT assay). A significant toxicity of CLZn was observed when its concentration exceeded 2×10^{-5} M (Fig. 2). The CLZn concentration lethal for 50% of the cells (IC_{50}) was estimated as 6.9×10^{-5} M. On the other hand, CLZm showed little toxicity up to the highest concentrations tested (10^{-4} M). Higher concentrations could not be tested, since 10^{-4} M is close to the maximal solubility of both compounds in the culture medium.

Concentration-Dependent Protection of t-BHP-Treated Hepatocytes by CLZn, CLZm, and Reference Antioxidants

The oxidative stress induced by 2.5×10^{-4} M *t*-BHP proved to be highly toxic to the cells, since about ninety percent of the hepatocytes died within 6 hr. The addition of low concentrations (3×10^{-6}–10^{-5} M) of CLZn and CLZm significantly protected the cells (Fig. 3). At these doses, CLZn improved the cellular survival from 12% in cells treated with *t*-BHP alone to more than 50% in cells incubated with 6.6×10^{-6} M CLZn. However, a strong toxicity occurred when higher CLZn doses ($>10^{-5}$ M) were applied. The synthetic analogue CLZm, while very effectively protecting hepatocytes even at the lowest concentration tested (3×10^{-6} M), maintained the survival in the 70–80% range as its concentration was raised to 30 µM. Nevertheless, the protection plummeted to zero at the highest CLZm concentration tested (5×10^{-5} M). While these results demonstrate that IMPZs can protect hepatocytes against the lethal effects of *t*-BHP, they also reveal that the above-described toxicity of CLZn is increased in the presence of *t*-BHP; on the other hand, CLZm, although not toxic when applied alone, exerts some detrimental action in coincubation with the oxidising stressor. Owing to the high toxicity of CLZn, further tests were only carried out with CLZm.

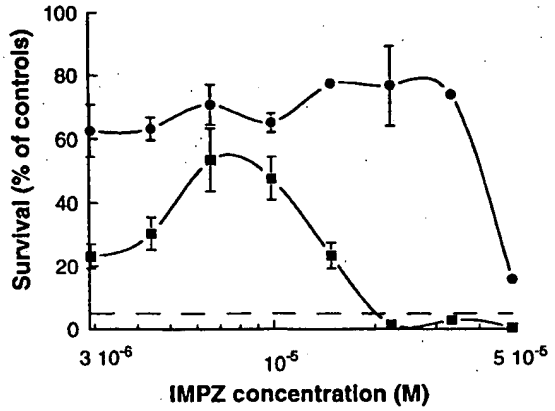


FIG. 3. Concentration-dependent effect of CLZn (■) or CLZm (●) on the survival of rat hepatocytes treated for 6 hr with 2.5×10^{-4} M *t*-BHP. Survival was assayed by the MTT test. Values are expressed as means ± SE of 6 replicates. Controls (100% survival) were treated with culture medium (DMEM) only.

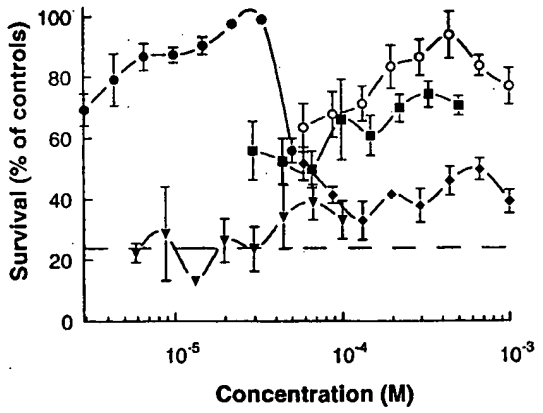


FIG. 4. Concentration-dependent protective effect of CLZm and reference antioxidants on the survival of rat hepatocytes treated for 6 hr with t-BHP (2.5×10^{-4} M). Survival was assayed by the MTT test. Values are expressed as means ± SE of 3 replicates. Controls (100% survival) were treated with culture medium (DMEM) only. The dotted line indicates the survival of cells treated with t-BHP alone. CLZm (●), Trolox C® (○), BHT (■), probucol (▼), α-tocopherol (◆).

In order to establish the relative antioxidative potency of this compound comparative to other well-known antioxidants, different concentrations of CLZm, α-tocopherol, BHT, probucol, and Trolox C® were tested on t-BHP-treated hepatocytes. The concentration range of each antioxidant was determined from doses reported to be effective in the literature and according to their respective solubility in the culture medium. Each antioxidant was coincubated with 2.5×10^{-4} M t-BHP for 6 hr. The survival was then measured by both the MTT cytotoxicity assay (Fig. 4) and the release of LDH into the culture medium (Fig. 5). The survival of t-BHP-treated hepatocytes was estimated at 24 ± 3% by MTT assay. A significant concentration-dependent protection was provided between 3 and 20 micromolar CLZm. A total protection was achieved at 2×10^{-5} M CLZm. At higher concentrations, however, this protective effect decreased, although cell survival remained higher than for the peroxide-treated cells without CLZm. Reference antioxidants were much less efficient cell protectors than CLZm. Except for Trolox C®, the hydrophilic analogue of vitamin E, none achieved a full protection such as obtained with CLZm. In the case of Trolox C®, it is noteworthy that a similar protection level was reached, but only at doses 100 times higher than those required for CLZm. BHT, like Trolox C®, significantly protected the cells at all concentrations tested (3×10^{-5} M to 5×10^{-5} M, maximal survival: 74.4 ± 4.3%). The protection conferred by α-tocopherol, although statistically significant ($P < 0.05$), remained very low. Probucol was not effective except at 6.7×10^{-5} M, where it slightly increased cell survival to 39.0 ± 5.8%.

Although survival levels estimated by LDH released into the supernatant were higher than those obtained with the MTT test, probably resulting from the additional two hours required in the MTT-based assay, similar conclusions could be drawn: CLZm achieved total cellular protection, followed by Trolox C® and BHT (Fig. 5). Probucol and α-tocopherol were less effective. It is important to note that the increased release of LDH observed with the highest concentration of CLZm (5×10^{-5} M) was only detected in the presence of t-BHP. CLZm as such did not significantly increase the LDH level in the culture medium (not shown).

Protection of Cellular Lipids Against Oxidation

t-BHP induced mortality was previously ascribed to its ability to trigger the peroxidation of membrane lipids [11]. Therefore, the improvement in cellular survival by IMPZs suggests that they were able to protect cellular lipids against the peroxidising effect of t-BHP. We thus examined whether CLZm could decrease the level of lipid peroxidation during a stress induced with 2.5×10^{-4} M t-BHP. Cellular lipid peroxidation in hepatocytes was estimated by the measurement of TBARS in the culture medium and in the cells (Table 1). Trolox C® was chosen as reference as it gave the best cytoprotection among antioxidants tested in the above experiment. CLZn was also included in this test to determine whether its toxicity could be related to some pro-oxidant effect, as shown for other antioxidants [12]. Control cells exhibited low TBARS values (277.38 ± 27.78 pmol/10^6 cells), with 85% of the signal being recorded in the culture medium. A 6-hr treatment of hepatocytes with 2.5×10^{-4} M t-BHP increased TBARS levels in the culture medium about 6-fold, while that of the cells was doubled. Consequently, 96% of the t-BHP-induced TBARS production was associated with the culture medium. Coincubation of the hepatocytes with CLZm (10^{-5} M and 5×10^{-5} M) reduced malondialdehyde (MDA) production in cells and medium to levels equivalent to

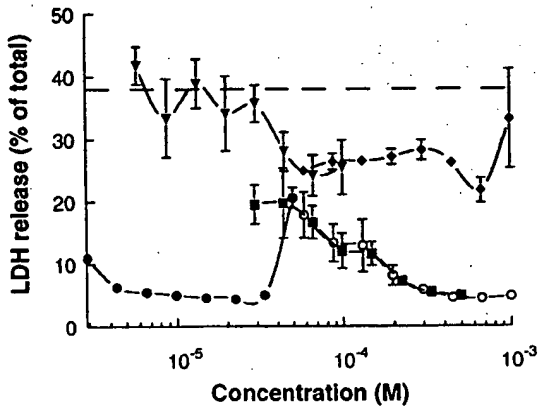


FIG. 5. Effect of CLZm and reference antioxidants on the release of LDH by hepatocytes treated for 6 hr with 2.5×10^{-4} M t-BHP. LDH values are expressed as means ± SE of 3 replicates. Total LDH content was estimated by treating the cells with Triton X-100 2%. The dotted line indicates the percentage of total LDH released by cells treated with t-BHP alone. CLZm (●), Trolox C® (○), BHT (■), probucol (▼), α-tocopherol (◆).

TABLE 1. Effects of antioxidants on TBARS production by t-BHP-treated rat hepatocytes

	TBARS (pmol malondialdehyde/well)		
	Cells	Medium	Total
Control cells	38.58 ± 7.40 a	238.80 ± 26.11 a,c,d	277.38 ± 27.78 a,c,d
t-BHP alone	84.87 ± 3.45 b	1366.69 ± 90.78 b	1451.57 ± 91.06 b
+ CLZn (5.10^{-5} M)	25.60 ± 4.02 a	353.03 ± 28.53 a,d	378.63 ± 31.59 a,d
+ CLZn (10^{-5} M)	26.55 ± 2.63 a	217.02 ± 37.68 c	243.57 ± 35.14 c
+ CLZm (5.10^{-5} M)	22.44 ± 2.26 a	86.86 ± 7.44 c	109.30 ± 9.35 c
+ CLZm (10^{-5} M)	25.74 ± 2.62 a	133.34 ± 16.74 c	161.64 ± 15.73 c
+ Trolox C (10^{-3} M)	21.54 ± 2.86 a	122.98 ± 2.76 c	144.52 ± 5.08 c
+ Trolox C (5.10^{-4} M)	27.25 ± 1.92 a	129.36 ± 7.86 c	156.61 ± 9.54 c
+ Trolox C (10^{-4} M)	37.87 ± 3.95 a	261.12 ± 8.30 c,d	286.37 ± 8.30 c,d
+ Trolox C (5.10^{-5} M)	37.27 ± 1.24 a	439.62 ± 59.96 d	464.47 ± 52.62 d

Cells (10^6/well) were treated for 6 hr with *t*-BHP (2.5×10^{-4} M) in the presence or absence of the tested compounds; TBARS were assayed in cells and in the culture medium. Mean ± SE, N = 3. Within each column, data not sharing the same letter are significantly different ($P < 0.05$).

those of untreated cells ($P < 0.05$). CLZm proved more efficient than CLZn, although the latter significantly reduced TBARS to levels not different from controls. Trolox C® also reduced TBARS levels in cells and the medium significantly. However, 5×10^{-5} M Trolox C® was significantly less effective than CLZm at the same concentration, with total TBARS of 464.47 ± 52.62 and 109.30 ± 9.35 pmol MDA/10^6 cells, respectively ($P < 0.05$). Higher Trolox C® concentrations (10^{-4}, 5×10^{-4}, 10^{-3} M) reduced TBARS to levels not significantly different from those recorded for the two CLZm concentrations. In order to rule out that reductions of TBARS in the supernatant could be related to some protection of the culture medium, instead of the cells, by antioxidants, preincubation experiments were performed. In these tests, cells were incubated with CLZm (10^{-5} M) or Trolox C® (10^{-3} M) for 1 hr, then medium was then removed, and the cells rinsed thrice with PBS. The peroxide was then applied on the cells. After 6 hr, TBARS were assayed in the supernatant and compared to those recorded for cells treated with *t*-BHP in the absence or the continued presence of the antioxidants. Cells previously loaded with 10^{-5} M CLZm showed TBARS levels corresponding to 20.76 ± 2.86% of those recorded for cells exposed to *t*-BHP alone. For Trolox C® (10^{-3} M), TBARS were reduced to 14.05 ± 2.15%. When cells were coincubated with *t*-BHP and either CLZm or Trolox C®, TBARS in the supernatants were 3.86 ± 0.52% and 2.82 ± 0.40%, respectively, that of the TBARS recorded for *t*-BHP alone. All these results indicated that the decreased TBARS levels in the presence of the antioxidants reflect a protective action on the cellular constituents whose oxidation generates TBARS in the medium rather than on the culture medium itself.

DISCUSSION

The results presented herein demonstrate that CLZm is able to protect rat hepatocytes against the lethal effects of *t*-BHP. It markedly improved cellular survival and helped to maintain the integrity of cellular membranes as revealed by the reduction of LDH and TBARS release. *t*-BHP has often been used as a model compound to study the effects of hydroperoxides in biological systems [11, 13–16]. Previous studies revealed that this oxidant, when used at concentrations such as employed in the present work, damages hepatocytes through the induction of lipid peroxidation [17, 18]. The mechanisms involved in the oxidation-dependent toxicity of *t*-BHP in hepatocytes have been linked to the ability of these cells to metabolise the hydroperoxide through two pathways [19]. First, *t*-BHP can be detoxified into its corresponding alcohol by glutathione peroxidase, thus depleting GSH stores. As an alternative pathway, *t*-BHP can be activated to radical intermediates by cytochromes P450 catalysing its cleavage into alkoxyl and peroxyl radicals [18, 20, 21]. These radicals can then initiate and propagate lipid peroxidation in cells whose susceptibility towards oxidative stress is increased through the first detoxification pathway.

The initiation of lipid peroxidation by *t*-BHP is known to occur intracellularly within the hydrophilic regions of membranes [22]. This has been explained by the hydrophilic nature of this hydroperoxide as well as by the organisation of the CYP 450 system tightly bound to the membrane of the endoplasmic reticulum, facing the cytosolic (external) side of the membrane [23, 24]. In such a cellular stress model, previous studies have shown that the extent of cellular protection provided by extracellularly applied antioxidants is conditioned by three main parameters: their entry into cells through the plasma membrane, their intracellular localisation near the sites where ROS are being generated and, finally, their ability to quench ROS [25].

Our results obtained with different reference antioxidants clearly illustrated the influence of these three parameters. Considering the diffusibility into cells, probucol has been shown not to cross plasma membranes [26, 27] and it provided no protection. On the contrary, BHT is a small hydrophobic phenol that penetrates easily into lipid bilayers, and it performed rather well in hepatocytes [28]. The efficiency of Trolox C®—a negatively charged compound

with a low diffusibility through membranes—demonstrates that this could be compensated for by the other two parameters. Nevertheless, the maximal efficiency of Trolox C® required millimolar concentrations [29–31]. In our experiments, and as already described by others [32], Trolox C® and BHT performed similarly. It is known that Trolox C® is much more reactive with ROS than BHT [28]. Therefore, the higher diffusibility of the phenol may compensate for its lower reactivity. The efficiency of Trolox C® may also be explained by its appropriate location in cells. Indeed, Trolox C® is relatively hydrophilic and lipophobic, partitioning about 20% into the lipid phase of liposomes [33]. This allows reactions of Trolox C® with radicals generated from *t*-BHP both in hydrophilic environments and also during the propagation of the lipid peroxidation process in the outer part of the membranes. The very limited protection conferred by α-tocopherol, which presents a similar reactivity with ROS to that of Trolox C®, may very well result from a limited availability at the majority of oxidation sites [34]. Furthermore, α-tocopherol is probably too lipophilic and less mobile, thus localising too deeply within the membranes [35], a situation which would not be appropriate in a *t*-BHP-induced oxidative stress.

The above dose–response experiments revealed that CLZm is about 10- to 100-times more potent than the most efficient reference antioxidants used in this study. According to what has been said above, CLZm is superior overall in terms of penetration into cells, localisation near sites under oxidative threat, and a high reactivity with the ROS involved in the cytotoxic effects of *t*-BHP.

There is no doubt that CLZm easily diffuses into cells. Indeed, CLZn, whose structure and hydrophobicity is very similar to CLZm, has been shown to readily access all cellular compartments such as the cytosol, the nucleus, and the endoplasmic reticulum [36, 37]. Our data indicate that CLZm also instantaneously enters rat hepatocytes,* and preincubation experiments in the present study confirmed that CLZm can rapidly associate to hepatocytes and protect cellular lipids against oxidation. Localisation of IMPZs could be optimal as well: IMPZs are amphiphilic compounds that should reach most sites where the peroxidative process occurs. Thanks to this distribution, they may counteract initiation reactions, scavenging radicals formed within the hydrophilic area of the membranes, and also act on the propagation of the chain reaction within the hydrophobic membrane.

Acellular experiments have shown that IMPZs are very good scavengers of a wide range of ROS, such as singlet oxygen and superoxide anions [3, 38]. Recently, CLZn has been shown to react with peroxynitrite [39]. CLZn also inhibits linoleate peroxidation induced by the hydrophilic free radical initiator 2,2'-azobis (2-amidinopropane)hydrochloride [4]. MCLA (2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-α]pyrazin-3-one, whose structure is very similar to that of CLZn and CLZm, was demonstrated to have antioxidant properties against lipid peroxidation [40]. The higher protective potency of CLZn compared to Trolox C® in delaying the acellular oxidation of linoleate suggests that the imidazolopyrazinone reacts faster with radicals generated in the peroxidative process [4]. Therefore, it is likely that the marked superiority of CLZm over other antioxidants in hepatocytes is also to be ascribed to a very high reactivity with a wide range of ROS generated intracellularly.

As another possibility to explain the superiority of IMPZs, one cannot *a priori* exclude an inhibitory action of CLZn and CLZm on some CYP 450 isoforms involved in the activation of *t*-BHP. Two mechanisms may be envisaged. First, a non-competitive inhibition could take place, such as described for imidazoles [41]. If such an inhibition occurs, this would decrease the activation of *t*-BHP and consequently reduce both lipid peroxidation and cell killing induced by the stressor. A second mechanism could take place if IMPZs are substrates of certain isoforms of CYP 450. In that case, the addition of *t*-BHP could increase the hydroxylation rate of imidazolopyrazinones, using CYP 450 as a peroxidase-like catalyst [42, 43], thus diverting CYP 450 from its radical-forming metabolic pathway. As a consequence, lipid peroxidation and cellular mortality would also be decreased. Possible actions of IMPZs on CYP 450s are currently being investigated.

The cellular toxicity of CLZn is intriguing. TBARS experiments carried out with cytotoxic concentrations of CLZn showed that lipid peroxidation values remained very low even though cells were dying, suggesting that the toxicity of CLZn does not involve a pro-oxidant effect. Moreover, another experiment revealed that Trolox C® (10^{-3} M), while protecting cells against *t*-BHP, did not reduce but even strongly increased the toxicity of CLZn (results not shown). The observation that the toxicity of CLZn and CLZm is increased or triggered, respectively, when *t*-BHP is being concomitantly applied suggests that their lethal action could result from their possible activation by CYP P450. CLZn and CLZm could then possibly be transformed into products harmful to the cell. As discussed above, the presence of *t*-BHP could increase the metabolism of IMPZs by CYP 450 as described with other compounds and thus act synergistically on cell survival [42, 43]. Accordingly, the higher tolerance of hepatocytes for CLZm could reflect a lower toxicity of metabolites or a lower rate of transformation into harmful by-products.

One may conclude from these data that CLZm, but not the natural CLZn, could be a good model compound for further studies of the mechanisms of this family of powerful antioxidants in rat hepatocytes.

* Dubuisson MLN, de Wergifosse B, Marchand-Brynaert J, Trouet A and Rees JF, manuscript in preparation.

This work was supported by a FIRST program of the Walloon Government to A. T. (Convention 2357) and by the National Fund

for Scientific Research (FNRS) of which J.F.R. and M.D. are Senior and Assistant Researchers, respectively. B.D.W. was supported by a grant from the Fonds pour la Recherche Industrielle et Agronomique (FRIA).

References

1. Thompson EM and Rees JF, Origins of luciferins: Ecology of bioluminescence in marine fishes. In: *Biochemistry and Molecular Biology of Fishes* (Eds. Hochachka PW and Mommsen TP), Vol. 4, pp. 435–466. Elsevier Science, Amsterdam, 1994.
2. Shimomura O, Presence of coelenterazine in non-bioluminescent marine organisms. *Comp Biochem Physiol* 86B: 361–363, 1987.
3. Suzuki N, Ogawa K, Yoda B, Nomoto T, Inaba H and Goto T, Chemiluminescent detection of active oxygen species, singlet molecular oxygen and superoxide, using *Cypridina* luciferin analogs. *Nippon Suisan Gakkaishi* 57: 1711–1715, 1991.
4. Rees JF, de Wergifosse B, Noiset O, Dubuisson M, Janssens B and Thompson EM, The origins of marine bioluminescence: Turning oxygen defence mechanisms into deep-sea communication tools. *J Exp Biol* 201: 1211–1221, 1998.
5. Poli G, Liver damage due to free radicals. *Free Radicals in Medicine. The British Council*: 604–620, 1993.
6. Chance B, Sies H and Boveris A, Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527–605, 1979.
7. Inoue S, Sugiara S, Kakoi H, Hashizume K, Goto T and Iio H, Squid bioluminescence II. Isolation from *Watasenia scintillans* and synthesis of 2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one. *Chem Lett* 1975: 141–144, 1975.
8. Seglen PO, Preparation of isolated rat liver cells. *Methods Cell Biol* 13: 29–83, 1976.
9. Wey HE, Pyron L and Woolery M, Essential fatty acid deficiency in cultured human keratinocytes attenuates toxicity due to lipid peroxidation. *Toxicol Appl Pharmacol* 120: 72–79, 1993.
10. Günther T, Vormann J and Höllriegl V, Effects of magnesium and iron on lipid peroxidation in cultured hepatocytes. *Mol Cell Biochem* 144: 141–145, 1995.
11. Tseng TH, Wang CJ, Kao ES and Chu HY, Hibiscus procatechuic acid protects against oxidative damage induced by *tert*-butylhydroperoxide in rat primary hepatocytes. *Chem Biol Interact* 101: 137–148, 1996.
12. Dyatlov VA, Makovetskaia VV, Leonhardt R, Lawrence DA and Carpenter DO, Vitamin E enhances Ca^{2+}-mediated vulnerability of immature cerebellar granule cells to ischemia. *Free Radic Biol Med* 25: 793–802, 1998.
13. Fernandes ER, Carvalho FD, Remiao FG, Bastos ML, Pinto MM and Gottlieb OR, Hepatoprotective activity of xanthones and xanthonolignoids against *tert*-butylhydroperoxide-induced toxicity in isolated rat hepatocytes—comparison with silybin. *Pharm Res* 12: 1756–1760, 1995.
14. Hwang JM, Tseng TH, Hsieh YS, Chou FP, Wang CJ and Chu CY, Inhibitory effect of atractylon on *tert*-butyl hydroperoxide induced DNA damage and hepatic toxicity in rat hepatocytes. *Arch Toxicol* 70: 640–644, 1996.
15. Miguez MP, Amundi I, Sainz-Pardo LA and Lindros KO, Hepatoprotective mechanism of silymarin: No evidence for involvement of cytochrome P450 2E1. *Chem Biol Interact* 91: 51–63, 1994.
16. Carini R, Comoglio A, Albano E and Poli G, Lipid peroxidation and irreversible damage in the rat hepatocyte model. Protection by the silybin–phospholipid complex IdB 1016. *Biochem Pharmacol* 43: 2111–2115, 1992.
17. Masaki N, Kyle ME and Farber JL, *tert*-Butyl hydroperoxide kills cultured hepatocytes by peroxidizing membrane lipids. *Arch Biochem Biophys* 269: 390–399, 1989.
18. Masaki N, Kyle ME, Serroni A and Farber JL. Mitochondrial damage as a mechanism of cell injury in the killing of cultured hepatocytes by *tert*-butyl hydroperoxide. *Arch Biochem Biophys* 270: 672–680, 1989.
19. Rush GF, Gorski JR, Ripple MG, Sowinski J, Bugelski P and Hewitt WR, Organic hydroperoxyde-induced lipid peroxidation and cell death in isolated hepatocytes. *Toxicol Appl Pharmacol* 78: 473–483, 1985.
20. Minotti G, *tert*-Butyl hydroperoxide-dependent microsomal release of iron and lipid peroxidation. *Arch Biochem Biophys* 273: 137–143, 1989.
21. Davies MJ, Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with rat liver microsomal fractions. *Biochem J* 257: 603–606, 1989.
22. Thornalley PJ, Trotta RJ and Stern A, Free radical involvement in the oxidative phenomena induced by *tert*-butyl hydroperoxide in erythrocytes. *Biochim Biophys Act* 759: 16–22, 1983.
23. Nakayama T, Hori K, Terazawa K and Kawakishi S. Comparison of the cytotoxicity of different hydroperoxides to V79 cells. *Free Radic Res Commun* 14: 173–178, 1991.
24. Lu AY, NADPH-dependent cytochrome P540 reductase. In: *Molecular Aspects of Monooxygenase and Bioactivation of Toxic Compounds* (Ed. Arinç E, Schenkman JB and Hodgson E), pp. 135–147, Plenum Press, NY, 1991.
25. Dean RT, Hunt JV, Grant AJ, Yamamoto Y and Niki E, Free radical damage to proteins: The influence of the relative localization of radical generation, antioxidants, and target proteins. *Free Radic Biol Med* 11: 161–168, 1991.
26. Pike CJ, Ramezan-Arab N and Cotman CW, β-amiloid neurotoxicity *in vitro*: Evidence of oxidative stress but no protection by antioxidants. *J Neurochem* 69: 1601–1611, 1997.
27. Roberts CP, Murphy AA, Santanam N and Parthasarathy S, Regulation of monocyte to macrophage differentiation by antiglucocorticoids and antioxidants. *Am J Obstet Gynecol* 179: 354–362, 1998.
28. Kaneko T, Kaji K and Matsuo M, Protection of linoleic acid hydroperoxide-induced cytotoxicity by phenolic antioxidants. *Free Radic Biol Med* 16: 405–409, 1994.
29. Massey K and Burton KP, Free radical damage in neonatal rat cardiac myocyte cultures: Effects of α-tocopherol, Trolox, and phytol. *Free Radic Biol Med* 8: 449–458, 1990.
30. Sugiyama H, Fung KP and Wu TW, Purpurogallin as an antioxidant protector of human erythrocytes against lysis by peroxyl radicals. *Life Sci* 53: PL 39–43, 1993.
31. Wu TW, Hashimoto N, Wu J, Carey D, Li RK, Mickle DA and Weisel RD, The cytoprotective effect of Trolox demonstrated with three types of human cells. *Biochem Cell Biol* 68: 1189–1194, 1990.
32. Lambert CR, Black HS and Truscott TG, Reactivity of butylated hydroxytoluene. *Free Radic Biol Med* 21: 395–400, 1996.
33. Ross L, Barclay C and Vinqvist MR, Membrane peroxidation: Inhibiting effects of water-soluble antioxidants on phospholipids of different charge types. *Free Radic Biol Med* 16: 779–788, 1994.
34. Decker DE, Vroegop SM and Buxser SE, Inhibition of oxidative insult in cultured cells by a novel 6-chromanol-containing antioxidant. *Biochem Pharmacol* 50: 1063–1070, 1995.
35. Castle L and Perkins MJ, Inhibition kinetics of chain-breaking phenolic antioxidants in SDS micelles. Evidence that intermicellar diffusion may be rate-limiting for hydrophobic inhibitors such as alpha-tocopherol. *J Am Chem Soc* 108: 6381–6382, 1986.

36. Kendall JM, Dormer RL and Campbell AK, Targeting aequorin to the endoplasmic reticulum of living cells. *Biochem Biophys Res Com* **189**: 1008–1016, 1992.
37. Kendall JM and Badminton MN, *Aequora victoria* bioluminescence moves into an exciting new area. *TIBTECH* **16**: 216–224, 1998.
38. Suzuki N, Suetsuna K, Mashiko S, Yoda B, Nomoto T, Toya Y, Inaba H and Goto T, Reaction rates for the chemiluminescence of *Cypridina* luciferin analogues with superoxide: A quenching experiment with superoxide dismutase. *Agric Biol Chem* **55**: 157–160, 1991.
39. Tarpey MM, White CR, Suarez E, Richardson G, Radi R and Freeman BA, Chemiluminescent detection of oxidants in vascular tissue. Lucigenin but not coelenterazine enhances superoxide formation. *Circ Res* **84**: 1203–1211, 1999.
40. Tampo Y, Tsukamoto M and Yonaha M, The antioxidant action of 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (MCLA), a chemiluminescence probe to detect superoxide anions. *FEBS Lett* **430**: 348–35, 1998.
41. Testa B and Jenner P, Inhibitors of cytochrome P-450s and their mechanism of action. *Drug Metab Rev* **12**: 1–117, 1981.
42. Minotti G, Borrello S, Palombini G and Galeotti T, Cytochrome P-450 deficiency and resistance to *t*-butyl hydroperoxide of hepatoma microsomal lipid peroxidation. *Biochim Biophys Acta* **876**: 220–225, 1986.
43. Hiroya K, Murakami Y, Shimizu T, Hatano M and Ortiz de Montellano PR, Differential roles of Glu318 and Thr319 in cytochrome P450 1A2 catalysis supported by NADPH-cytochrome P450 reductase and *tert*-butyl hydroperoxide. *Arch Biochem Biophys* **310**: 397–401, 1994.